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TITLE: Laser Raman Tweezer Spectroscopy for the Molecular and Functional Characterization of Single Live Mouse Mammary Tumor-Initiating Cells

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INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The purpose of this research is to utilize Raman Spectroscopy (RS) combined with optical tweezer technology (RTS) to isolate and characterize the intracellular molecular profiles of live tumor initiating cells derived from p53 null mouse mammary tumors. The rationale is that previous technology has used flow cytometry and antibodies to specific extracellular markers for the purpose of isolation and characterization of tumor initiating cells (TIC) or cancer stem cells (CSC). RTS would allow characterization of cells based on their intracellular molecular profiles. Combining the methods of flow cytometry that allows cell isolation by extracellular markers with RTS that enables the isolation of cells with distinct intracellular molecular profiles would allow one to distinguish heterogeneous cells among CSCs. This technology would enable one to identify cells with distinct intracellular molecular profiles among an otherwise homogeneous population of CSC and to translate the distinct intracellular molecular profiles to *in vitro* and *in vivo* biological functions such as tumorigenicity. The long-term goal of this project is the isolation and characterization of distinct tumor initiating cells; which would enable future development of anti-cancer stem cell therapies for eradicating resistant and or quiescent CSCs.

BODY:

Task 1. To determine the tissue culture condition, microscope lens setting, and laser intensity for obtaining Raman spectra of live single cells derived from p53 null mouse mammary tumors with minimal damage to the cells.

We initially used a breast cancer cell line, SUM225. The rationale was that SUM225 cells were large (15-30 micron) enough to be visualized by an upright microscope at various, 4 to 100 X, magnifications. Visualizing the cells at various magnifications enabled the optimization of laser intensity with minimal damage to the cells. We were able to obtain Raman spectra using a 100X lens of a confocal microscope submerged into the tissue culture medium and by the use of a HeNe laser (632.8 nm) excitation. The scattered Raman signal was detected on a Peltier-cooled, back-illuminated, deep depletion CCD chip (Andor, Northern Ireland) after passing a holographic transmissive grating. Integration time was 20 seconds repeated three times and the mapping interval was 2 microns. We established that tissue culture medium that contained phenol red and culture plates made of plastic interfered with Raman spectra. By switching to Quartz plates and using phosphate buffer saline (PBS), background Raman signal was minimized. As seen in **Figure 1**, we have been successful in obtaining Raman signals from a single SUM225 cell. **Figure 2** shows that there is no visible cell damage following a 45 minute Raman acquisition period using the HeNe laser illumination.

Task 2. To perform RS profiling of single live cells derived from p53 null mouse mammary tumors.

P53 mouse mammary tumors were obtained from Dr. Jeffrey M. Rosen's laboratory at Baylor College of Medicine, in Houston, Texas. The tumors were shipped to our laboratory frozen and over night. The tumors were digested for two hours per established protocols in the laboratory. Single cells were obtained and cultured on quartz dishes in the appropriate tissue culture medium. The cells were then transported to the Raman laboratory at the University of Kansas Lawrence for Raman spectroscopy. At the time of profiling, tissue culture medium was switched to PBS. Single live cells derived from p53 null mouse mammary tumors were excited using a HeNe laser at 2 micron resolution in the X and Y axis, and Raman spectral profiles were collected. We obtained RS profiles of five cells (**Figure 3**). As seen, indicated by the arrows, regions of heterogeneity in Raman shifts are observed a 1000, 1300, 1500 and 1700 cm^{-1} positions.

Task 3. To utilize RTS for isolating p53 null derived tumor initiating cells (TICs) with distinct intracellular molecular profiles. The TICs with distinct intracellular molecular profiles will be evaluated for their *in vitro* and *in vivo* tumorigenicity.

Figure 4 is a diagram describing our future plans for the isolation, molecular profiling by RS, and assessment of tumorigenicity of TICs derived from p53 null mouse mammary tumors. Please note that a Raman Tweezer technology is currently not available at our institution. We will perform RS as described in **Figure 4**. If not successful, we will seek help from other institution with a Raman Tweezer capability. As outlined in **Figure 4**, TICs are sorted by flow cytometry based on their unique extracellular expression of $Lin^{-}CD29^{hi}CD29^{hi}$ markers. TICs are sorted onto individual quartz discs that are submerged onto a quartz tissue culture plate in PBS. The quartz tissue culture plates containing submerged quartz discs are placed onto the microscope stage and Raman spectra are obtained. Raman spectra are collected and analyzed as described under **Task 4**. The individual groups of cells that exhibit distinct intracellular molecular profiles will be assessed for their *in vitro* and *in vivo* tumorigenicity. *In vitro* tumorigenic potential will be assessed by cell motility and invasion assessed by the modified boyden chamber assay, proliferation index by Edu incorporation and mammosphere assays. These assays are established methods used routinely in the laboratory. *In vivo* tumorigenicity will be also performed by cleared fat pad transplantation. It has been shown that cells in $Lin^{-}CD24^{hi}CD29^{hi}$ subpopulation generate tumors with a frequency of 1 in 27 cells (95% CI: 1/42-1/18) (Zhang, 2008). Therefore, various cell concentrations (50, 25, 10, 5, and 1) from 5-7 groups (that display distinct intracellular profiles) and a control group (randomly isolated) will be injected into cleared fat pads of 6 mice or 12 fat pads per cell dilution (225 mice total including 25% loss factor). Poisson statistics will be performed to calculate statistical differences in frequency of TIC. It is expected that at least one group will display higher TIC frequency compared to the total population of TIC ($Lin^{-}CD24^{hi}CD29^{hi}$).

Task 4. Data analysis

Principle component model (PCA) and hierarchical clustering of the data will be calculated as described in (Neugebauer, 2010) and illustrated in **Figure 5**. Each cluster represents a group of cells with a unique intracellular molecular profile. We expect to find 5-7 unique spectral profiles. Cells representing each group (profile) will be evaluated for *in vivo* and *in vitro* tumorigenicity as described under **Task 3**.

Expected Outcome and future directions

We expect to find 5 to 7 unique groups of cells with distinct Raman spectra among the p53 null mammary tumor derived TICs. We expect to find that the groups of cells with unique profiles to show variability with respect to *in vivo* and *in vitro* tumorigenicity. We expect to find that a homogeneous group of cells that show similarities with respect to extracellular markers, are indeed heterogeneous with respect to intracellular profiles and tumorigenic potential. Future genomic and proteomic characterization of cells with distinct Raman profiles may be used to find unique signaling pathways or genes underlying higher tumorigenic potential. These studies have the promise to facilitate future development of anti-cancer stem cell therapies for eradication of the most aggressive tumor initiating cells. Furthermore, combined flow cytometry and Raman spectroscopy may offer a better strategy for the identification and characterization of tumor initiating cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Oral Presentation at The University of Kansas Lawrence, Bioengineering Department, Lawrence, Kansas, April 12, 2012.
- We plan to submit a manuscript by the end of this project, April 2013.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- None at this time.

CONCLUSION:

We have shown that it is feasible to obtain Raman signals from individual live cells derived from p53null mouse mammary tumors. We have established that Raman signals may be obtained from cells that have adhered to a quartz tissue culture dish and are excited by a HeNe laser. Future goals are to obtain data from large quantities of sorted p53 null mouse mammary TICs. The data analysis by PCA and Hierarchical clustering will be performed to identify and characterize groups of cells with unique intracellular Raman profiles. We expect to find that the groups of cells with unique profiles to show variability with respect to *in vivo* and *in vitro* tumorigenicity. We expect to find that a homogeneous group of cells that show similarities with respect to extracellular markers, are indeed heterogeneous with respect to intracellular molecular profiles and tumorigenic potential. Future genomic and proteomic characterization of cells with distinct Raman profiles may be used to find unique signaling pathways or genes underlying higher tumorigenic potential. These studies have the promise to facilitate future development of anti-cancer stem cell therapies for eradication of the most aggressive tumor initiating cells. Furthermore, combined flow cytometry and Raman spectroscopy may offer a better strategy for the identification and characterization of TIC from various types of human tumors.

SUPPORTING DATA:

All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

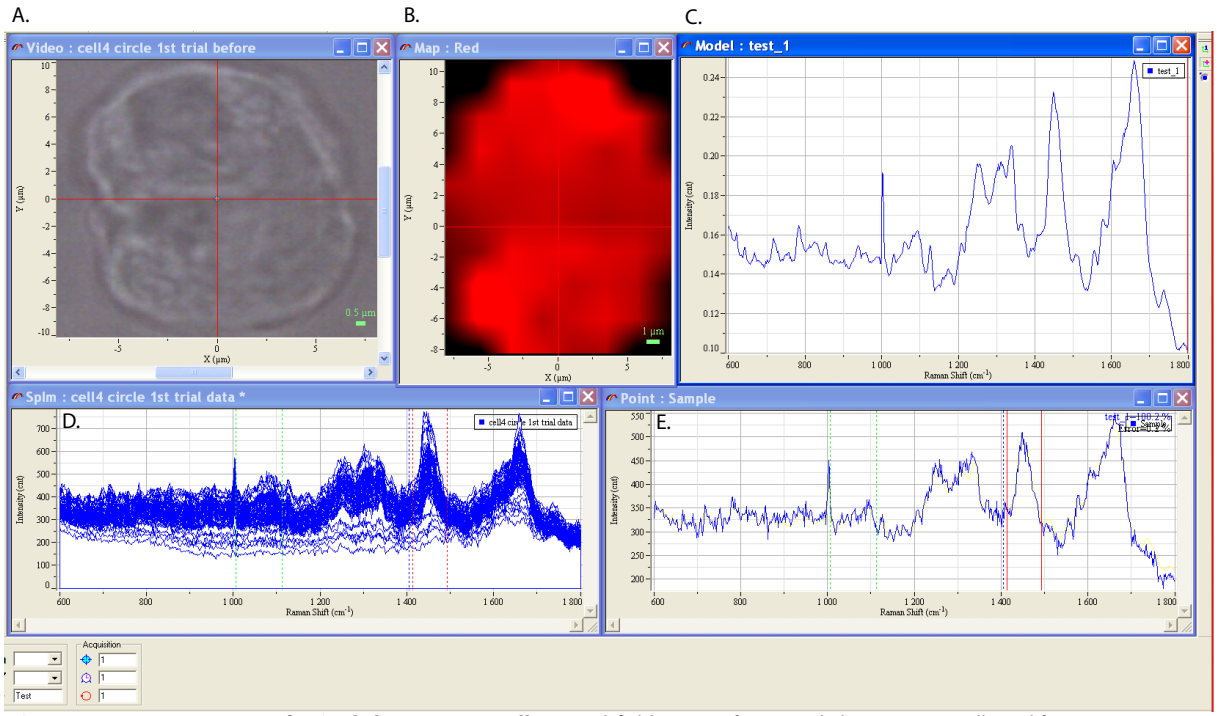


Figure 1. Raman Spectrum of a single live SUM225 cell. A. Brighfield image of one single live SUM225 cell used for acquiring Raman fingerprints. B. Raman cluster image obtained by scanning the confocal laser in a 10 x 10 raster pattern. The shades of red illustrates the spatial variation of Raman signal within a single cell. C. Raman shifts observed in the 600-1800 cm^{-1} regions. D. Cumulative Raman shifts following scanning of a single cell in a 10 x 10 raster pattern. E. Shows Raman shift of a single point in the center of the red corss in A.

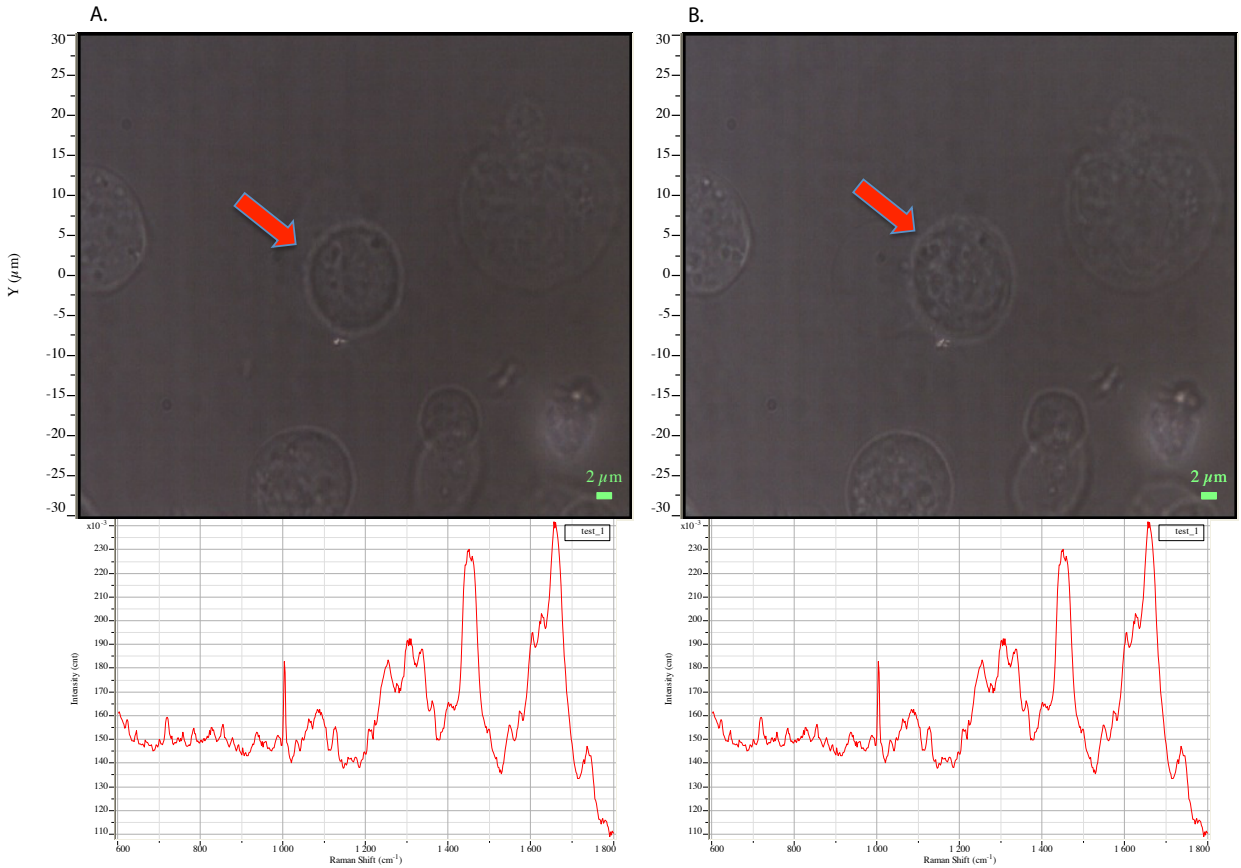


Figure 2. A representative averaged Raman spectrum of a single live SUM225 cell. A HeNe laster (632.8) is used for excitation at a 2 micron resolution over a 20 second dwell time. The arrows point to a brightfield image of a single live cell before (A) and after (B) obtaining Raman spectra. As seen, there is no visible sign of cell damage or change in Raman Spectrum following a 45 minute Raman acquistion period.

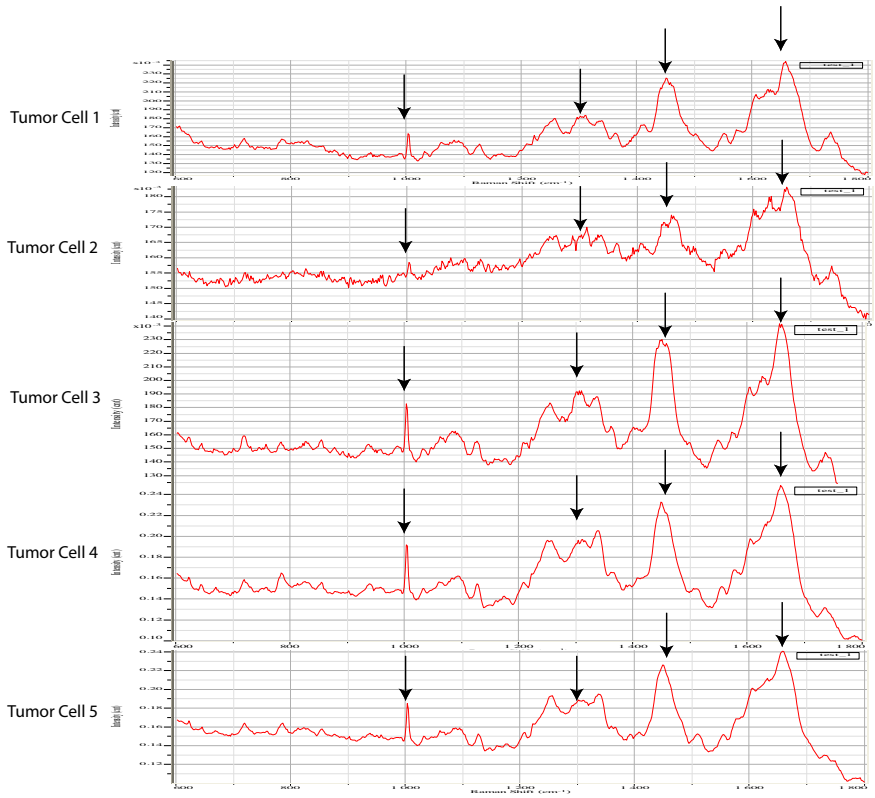
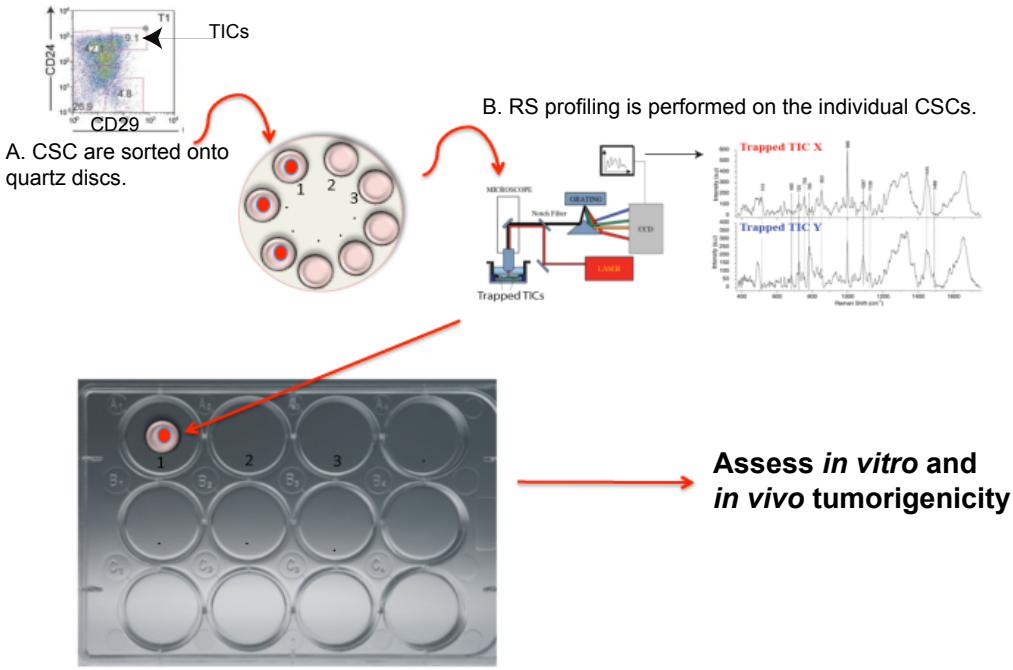


Figure 3. Raman shifts observed in five cells derived from p53 null mouse mammary tumor cells. Arrows indicate regions of heterogeneity among the tumor cells at 1000, 1300, 1500 and 1700 cm^{-1}.



C. Individual cells (red dots) are transfered onto a tissue culture plate and assessed for tumorigenicity.

Figure 4. Plans for the isolation and characterization of p53 null mouse mammary tumor initiating cells (TIC) by Raman Spectroscopy. A. P53 null tumor derived TIC are sorted by flow cytometry. TICs are identified by the expression of Lin-CD24hiCD29hi markers (indicated by arrow to the left). TICs are sorted onto individual quartz discs that are submerged onto a tissue culture plate in PBS. B. The quartz tissue culture plates containing the submerged quartz discs are placed on the microscope stage and Raman spectra are obtained. C. Following RS profiling, the discs are transferred onto a 12 well tissue culture plates followed by in vitro and in vivo tumorigenic assessment.

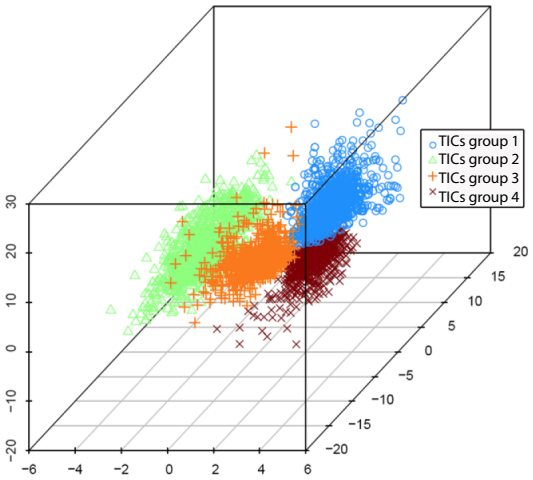


Figure 5. A hypothetical plot of principle component analysis of Raman spectral data from groups of TICs with four distinct intracellular molecular profiles (adopted from Neugebauer Ute et al, J Biophoton, 2010).

REFERENCES:

Neugebauer, U., Clement, J. H., Bocklitz, T., Krafft, C., and Popp, J. (2010). Identification and differentiation of single cells from peripheral blood by Raman spectroscopic imaging. J Biophotonics *3*, 579-587.

Zhang, M., Behbod, F., Atkinson, R. L., Landis, M. D., Kittrell, F., Edwards, D., Medina, D., Tsimelzon, A., Hilsenbeck, S., Green, J. E.*, et al.* (2008). Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. Cancer Res *68*, 4674-4682.

APPENDICES:

-N/A